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# IDENTIFICATION OF NATIVE MYCOBACTERIUM SMEGMATIS CELL WALL DEGRADATIVE ENZYMES USING ELECTROPHORETIC FLUORESCENT ASSAYS

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## ABSTRACT

Mycobacterium is an important bacterial genus containing many serious pathogens, including *Mycobacterium tuberculosis*. The mycobacterial cell wall is thick, rigid, waxy and extremely important for its survival in a hostile environment. Mycobacterium species contain multiple cell wall hydrolases, which are critical for cell growth, cell division and latent cell reactivation. Additionally, cell wall hydrolases can be considered as a group of potential drug targets and therapeutic agents for mycobacterial pathogens. However, the conventional hydrolytic enzyme assays are slow, laborious and inaccurate. In this study, we used a fast-growing, non-pathogenic bacterial strain - *Mycobacterium smegmatis* - to develop a more sensitive and efficient assay. With this assay, we were able to simultaneously quantify the enzymatic activity and localize the enzymatic protein bands. Furthermore, this new assay speeds up the purification and identification of native cell wall hydrolases. In this study we isolated three proteins showing hydrolytic activity with estimated molecular masses of 30 kDa, 70 kDa and 110 kDa. Overall, the knowledge obtained from this study should be translated to clinical research and ultimately used for the diagnosis and treatment of mycobacterial pathogens, including *M. tuberculosis*.

**KEYWORDS:** Mycobacterium smegmatis, M. tuberculosis, hydrolase, cell wall degradation enzymes, cell wall hydrolase assays, zymography.

## INTRODUCTION:

The genus *Mycobacterium* is comprised of many significant pathogenic species that cause worldwide human mortality and morbidity. One of its most well-known and studied members, *Mycobacterium tuberculosis*, is the causative agent of tuberculosis (TB) in both animals and humans. TB is one of the world's deadliest diseases, making it a major threat to public health. Although the incidence of TB has slowly declined over the past decade, an estimated 1.3 million deaths occurred in 2012 (Sulis et al., 2014). One of the reasons why *M. tuberculosis* has been so difficult to eliminate is because of the structure of its cell wall. The mycobacterial cell wall is responsible for maintaining the integrity and turgor pressure of the cell. The structure of the mycobacterial cell envelope is highly complex and unique compared to other bacteria (Brennan 2003; McNeil and Brennan, 1991). This is because it features a very thick, waxy, hydrophobic, and impermeable cell wall that is at least partially responsible for its resistance to numerous drugs (Hett and Rubin, 2008). Additionally, mycobacteria also contain cell wall hydrolases, which are a group of enzymes that cleave the cell wall network, and presumably play an important role in cell growth, cell elongation, daughter cell separation and latent cell resuscitation (Mukamolova et al.,1998; Tufariello et al., 2004). Consequently, uncontrolled hydrolytic activity by cell wall hydrolases can induce damage that may cause the bacterial cell wall to burst or increase its permeability to antimicrobial agents (Shockman 1992; Chao et al., 2013).

Unlike *M. tuberculosis*, *Mycobacterium smegmatis* is a non-pathogenic and fast-growing mycobacteria strain (3 to 4 hour doubling time), which makes it an ideal candidate for molecular and biochemical experiments in a biosafety level 2 facility. In addition, *M. smegmatis* contains several dozen cell wall hydrolases in its genome that are mostly homologous to those found in *M. tuberculosis*. Based on the homology of known hydrolase sequences in other bacteria, these gene sequences were identified using the mycobacterial genome and cloned. In this way, a recombinant cell wall hydrolase, Rv3915 (CwlM), was encoded from the *M. tuberculosis* H37Rv gene and reported in 2005 (Deng et al., 2005). This was followed by Rv1477 (RipA), Rv1009 (RpfB), Rv2450c (RpfE) and others (Hett et al., 2007; Hett et al., 2008; Mukamolova et al., 2006; Xue et al., 2012). Many hydrolase genes have been found to be "essential genes" for an organism's survival, indicating that these enzymes play critical roles in bacterial growth, division, and pathogenesis (Sassetti et al., 2003; Downing et al., 2004). However, the activity of tagged recombinant enzymes is relatively low in a cell-free system due to their altered conformations (lack of native folding). Therefore, it is very difficult to assess the specific activity of a particular gene product and to compare the catalytic activities among different recombinant enzymes.

Due to their role in bacterial division and in disrupting the integrity of the bacterial cell, mycobacterial hydrolases can potentially be used to (1) inhibit growth and division, (2) render the pathogen suicidal, or (3) increase the permeability of the bacterial envelope to antimicrobial drugs. Therefore, it is hypothesized that mycobacterial cell wall hydrolases can be exploited as a novel target for anti-mycobacterial agents in the treatment of mycobacterial pathogens, including *M. tuberculosis*. In recent years, putative hydrolase genes were identified using genomic data, whereas native cell wall hydrolase activity was detected from *M. smegmatis* in earlier studies (Kilburn et al., 1977; Deng et al., 1995; Li et al., 1996). However, overall progress has been slow due to the complicated nature of the hydrolases and a lack of efficient enzymatic assays. Currently, hydrolase activity measurement relies on turbidity monitoring and zymography methods that are inaccurate, require more substrate materials and laborious to perform.

In this study, a new and more efficient fluorescent assay was developed that can detect the activity of mycobacteria hydrolases (or cell wall degradative enzymes). Using this new assay, three major native cell wall hydrolases were identified with estimated molecular masses of 30 kDa (Hydrolase-30), 70 kDa and 110 kDa. This new assay and the early discovery of native cell wall hydrolytic enzymes will facilitate the purification of each enzyme and the identification of the gene it is encoded by. Overall, this new assay will accelerate basic research in mycobacteria and ultimately contribute to the fight against TB.

## MATERIAL AND METHODS:

### Preparation and purification of insoluble mycobacterial cell wall:

*M. smegmatis* ($MC^2155$) cells were lysed by extensive grinding in liquid nitrogen and through the use of an ultrasonic probe (Branson). The total cell lysate was thoroughly washed with 30 mL of 20 mM Tris–HCl buffer pH 7.9 and then centrifuged (4400 x g) to form a pellet. The pellet was further purified with an overnight treatment with DNase (0.1 mg/ml) and RNase (0.1 mg/ml) at 37°C. The pellet was then incubated with 0.1% (w/v) trypsin overnight twice, and finally with 0.1% (w/v) proteinase K overnight twice. The material was extensively washed five times with ethanol and then another five times with distilled water. Dialysis of the sample was performed using a 3 mM Tris–HCl buffer pH 7.9 solution. Finally, the insoluble material was lyophilized and was referred to as the insoluble cell wall (Deng et al., 2005).

### Conjugation of cell wall with fluorescein isothiocyanate (FITC):

The fluorescein isothiocyanate (FITC; Molecular Probes) labeled bacterial cell wall was prepared by covalently linking FITC to amide groups located in the cell wall. This conjugated FITC *M. smegmatis* cell wall was used as the substrate for all of the mycobacterial hydrolase assays. 20 mg of insoluble *M. smegmatis* cell wall was labeled with 1 mL of FITC for 16 hours in 10 mg/ml of Dimethylformamide (DMF; EM Sciences) and 0.3% of Triethylamine (TEA; EM Sciences). Unbound FITC was eliminated by pelleting the insoluble cell wall via centrifugation (10,000 x g) and the supernatant was removed. The product was then washed four times with DMF, four times with ethanol and four times with distilled water. During each wash, the product was centrifuged (10,000 x g) and then the supernatant was removed. The FITC-labeled cell wall pellet was dialyzed using distilled water and then lyophilized overnight (Krishnanurthy,

1999). Insoluble FITC conjugated *M. smegmatis* cell wall was used as a substrate for all of the mycobacterial hydrolase assays (including enzymes that specifically cleave components of the mycobacterial outer cell wall). This material could also be used to make 1% (w/v) agar plates and for phenotypic and cell free enzymatic assays, such as the lytic dot assay.

**Purification of hydrolases and determination of the hydrolase activity by fluorescent lytic dot assay:**
The crude enzyme sample was fractionated by fast protein liquid chromatography (FPLC) using a Bio-Scale Q-column (anionic exchange column). The fractions were then collected and dialyzed. The desalted fractions were assayed using either the fluorescent lytic dot assay or via fluorescent spectrophotometry. The fractions that displayed lytic activity were further purified using either a size exclusion column or size filtration centrifugal column. The fluorescent lytic dot assay is similar to the general lytic dot assay, except 0.1 - 0.5 mg/ml of insoluble FITC conjugated *M. smegmatis* cell wall was used as the matrix for the gel (Deng et al., 2005). After enzymatic incubation at 37°C overnight, the fluorescent dot(s) were directly observed under ultraviolet (UV) light.

**Identification of the hydrolase protein by electrophoretic fluorescent lytic assay:**
The FITC conjugated *M. smegmatis* cell wall was incubated with enzyme samples that had different levels of purity. The above reaction mix was loaded onto a non-denaturing 8-16% polyacrylamide gradient mini gel and analyzed by electrophoresis in the absence of sodium dodecyl sulfate (SDS). The mini gels were purchased from Bio-Rad and contained 0.09 M Tris, 0.08 M boric acid, 2.6 mM EDTA, and 0.2% sodium azide. Tris, borate and EDTA (TBE) was used as the sample buffer and contained 0.2 M Tris-base, 0.2 M boric acid, and 20 mM EDTA with a pH of 8.3. Gels were run in running buffer containing 0.04% (w/v) CHAPS for approximately 1 hour at a constant 200 V. After electrophoresis, the gel cassette was incubated overnight at 37°C and then imaged under UV light. The migration of the fluorescent smear and fluorescent protein bands were measured. The molecular weight of the fluorescent protein band(s) was estimated using Kaleidoscope prestained protein standards (Bio-Rad).

**RESULTS:**
**Detection of cell wall hydrolase activity from total cell lysates and partially purified fractions by fluorescent assay**:
The cell free enzyme extracts was prepared using both *M. smegmatis* cell lysates and cell filtrates (bacterial liquid culture collected after filtration). The enzyme extracts were then incubated with *M. smegmatis* cell wall tagged with FITC. **Figure 1A** depicts the molecular structure of FITC, whereas **Figure 1B** displays an image of the FITC conjugated insoluble *M. smegmatis* cell wall taken under UV light. Native cell wall hydrolase activity was readily detected and measured using both a spectrophotometer and a fluorescent lytic dot assay. These two assays were more efficient and easier to perform compared to both the traditional turbidity and conventional lytic dot assays. Unlike the conventional lytic dot assay, the fluorescent lytic dot assay used very little matrix material (0.2-0.5 mg/mL instead of 5 mg/mL) or insoluble cell wall. In addition, it is much simpler perform because it did not involve the use of methylene blue staining and destaining. Instead, the enzyme fractions that had hydrolase activity (positive fractions) fluoresced under UV light and were easily visualized. The fluorescent lytic dot assay is very powerful because it was used to detect both partially and relatively purified protein fractions. Furthermore, the intensity of the fluorescence is proportional to the intensity of hydrolytic activity. **Figure 1C** represents an image of the fluorescent lytic dot assay taken under UV light. Row 1 contains the positive hydrolase fraction, Row 2 contains the fraction with mild hydrolase activity and Rows 3 and Row 4 (bottom) contain the negative fractions without any hydrolase activity. In order to identify specific native hydrolases, we incorporated classical chromatographic protein purification procedures. Under this approach, cell free enzyme extracts were first purified using fast protein liquid chromatography (FPLC) with an anionic-exchange column (Q-column). The extracts were then further purified using a size exclusion column (P-50). The hydrolase positive fractions were then easily identified using the fluorescent lytic dot assay.

**Distribution of hydrolytic activities in subcellular fragments of bacterial lysates by fluorescent assay:**
This new fluorescent assay can also be used to perform quantitative analysis. In an effort to understand and assess the distribution of the cell wall hydrolases in different subcellular locations (cytosol, cell wall and membrane, and the cell filtrates in the extracellular space), the *M smegmatis* culture was first harvested and then lysed. This was followed by fractionation, which further isolated the culture into the cytosol, and cell wall and membrane fractions. The cell filtrates were concentrated, dialyzed, and lyophilized. Each subcellular fraction was normalized to the same volume using a similar buffer and incubated with *M. smegmatis* FITC cell wall. Following a 75-minute incubation at 37°C, fluorescence was measured using a spectrophotometer with an excitation wavelength set to 495 nm and an emission wavelength of 519 nm. The results were summarized in **Table 1**, which showed that a significant amount of hydrolase activity (~ 36%) was found in the extracellular space. This indicated that several *M. smegmatis* cell wall hydrolases were presumably secreted into the extracellular space. Additionally, there was much lower hydrolase activity in the cytosol (~20%) compared with the fraction that contained both the membrane and cell wall (~43%). This was expected because the cell membrane and wall are areas that would normally be catalyzed by hydrolases.

**Identification of native hydrolase proteins by electrophoretic lytic fluorescent assay:**
For decades, native gradient polyacrylamide gel electrophoresis (PAGE) has been commonly used to analyze cell wall fragments and to profile charged polysaccharides. A green-brown smear can be visualized on the gel after it has been stained with methylene blue, alcian blue and silver. The migration of the smear corresponds with a range of the molecular masses of the polysaccharides (Deng et al., 2000). In this particular study, a reaction mixture containing *M. smegmatis* FITC cell wall and lysozyme (both active and inactive) was loaded onto a non-denaturing PAGE gel (**Figure 2A**). Lane 1 contained a mixture of *M. smegmatis* FITC cell wall and heat inactivated lysozyme that was incubated for 2 hours. Only a very faint smear can be detected because the inactivated lysozyme was unable to hydrolyze the insoluble cell wall, leaving it intact. Lanes 2-4 contained *M. smegmatis* FITC cell wall and lysozyme that were incubated for 10 minutes, 2 hours and 5 hours, respectively. A fluorescent smear was visualized in all 3 lanes, which indicated the release of the soluble polysaccharide due to cleavage of the insoluble cell wall by the lysozyme. The intensity of the cleavage was proportional to the incubation time. Thus, the longer the incubation time, the stronger the smear on the gel.

In addition, non-denaturing gradient PAGE is also the gold standard for separating protein fractions, while maintaining their activity. Protein bands are localized using either Coomassie Brilliant Blue or silver staining. However, silver staining will denature the target protein if it is an enzyme. Using this technique, we identified the specific mycobacterial cell wall hydrolases that were responsible for the hydrolytic activity by using another enzymatic assay. An electrophoretic lytic fluorescent assay was developed for *M. smegmatis* cell wall hydrolases that combine the above two procedures (fluorescent assay and 8-16% gradient PAGE gel). Each well was loaded with a mixture that contained a partially purified (through ion-exchange column chromatography and a size column) *M. smegmatis* enzyme fraction and *M. smegmatis* FITC cell wall. Unlike in **Figure 2A**, the gel was incubated at 37°C overnight after completion of electrophoresis. This procedure (shown in **Figure 2B**) allowed the protein bands (if the protein was a hydrolase) to contact the substrates and degrade the nearby soluble *M. smegmatis* FITC cell wall. When the *M. smegmatis* FITC cell wall was hydrolyzed, its quencher was removed allowing fluorescent light to be emitted. Because the enzyme is distributed in a horizontal position as a protein band, a fluorescent band was visualized. The number of fluorescent bands was proportional to the number of the hydrolases in each purified fraction. The migration distance of each band on the PAGE gel corresponded to its molecular mass. Lane 2 is a partially purified positive fraction that contained three fluorescent bands (as pointed out by the arrows) with molecular masses of 30 kDa, 70 kDa and 110 KDa. Lane 3 is a relatively more purified fraction and contained one major fluorescent band visualized at 30 kDa, which we tentatively named hydrolase-30. Lane 1 contained a negative fraction without any hydrolase activity (no bands). Overall, this was a more sensitive assay for the identification of cell wall hydrolase protein(s) and their corresponding activities.

**DISCUSSION:**
There are two ways to study a targeted enzyme (cell wall hydrolase). The first method requires the identification of a putative gene(s). Then, the putative gene(s) must be cloned and expressed to synthesize a recombinant enzyme. However, recombinant enzymes may or may not be very active due to 3-D conformational changes and secondary modifications. The second method requires the development of enzymatic assays to purify and characterize enzymes in their native form. Once the enzyme has been characterized, the genes that encode it are then identified. The second method is a more classical approach and has the advantage of maintaining the full hydrolytic activity of the enzyme. However, this approach can often be extremely laborious and time consuming.

In this study, we developed a new and more efficient fluorescent assay that can detect native mycobacteria hydrolases (or cell wall degradative enzymes) from both a quantitative and qualitative standpoint. Three major native cell wall hydrolases were simultaneously identified that had estimated molecular masses of 30 kDa (Hydrolase-30), 70 kDa and 110 kDa. Recent experiments have shown that Hydrolase-30 is a strong native hydrolase that can be used to damage the cell envelope of *M. smegmatis* (data not shown). Further refinement of the current enzyme assay will facilitate the discovery of more cell wall hydrolases, their presumed genes, and lead to more vigorous drug studies.

Although the ultimate objective is to study cell wall hydrolases in pathogenic *M. tuberculosis*, *M. smegmatis* was used in this study as a substitute. *M. smegmatis* is a fast-growing non-pathogenic mycobacterial species that is easy to grow in a biosafety level 2 laboratory. Additionally, the presumed gene sequences of *M. smegmatis* native hydrolases can be identified once the partial protein sequences or peptide fingerprints are elucidated through mass spectrometry. This will then lead to the identification of relevant *M. tuberculosis* hydrolase genes because most of the known *M. smegmatis* genes have high homology with those of *M. tuberculosis*. Cell wall hydrolases must also be further investigated to better understand the critical roles they place in normal mycobacterial growth and in pathogenesis. This would involve chemical characterization of hydrolase reac-

tions, subcellular localization of the enzymes and the role they play in gene expression and regulation. Nevertheless, this study provides the basic tools for more extensive studies of cell wall hydrolases in order to manipulate gene expression patterns as a new and more effective means of combatting mycobacterial pathogens.

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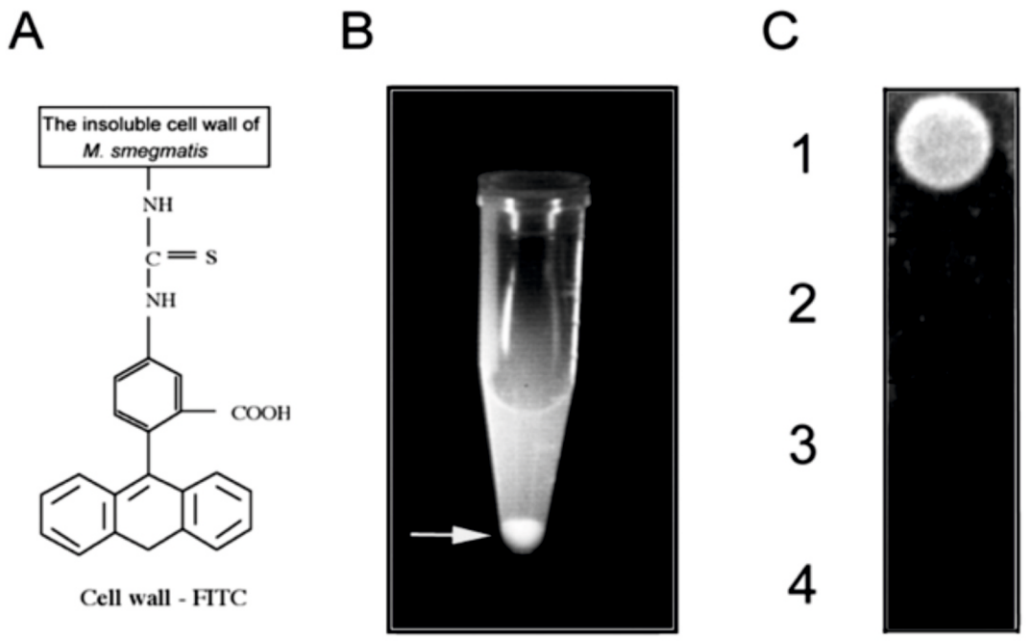


**Figure 1. Detection of native hydrolase activity by fluorescent assay.**

**A.** Structure of fluorescein isothiocyanate (FITC) that was conjugated to the M. smegmatis cell wall.

**B.** FITC conjugated insoluble M. smegmatis cell wall sample.

**C.** Fluorescent lytic dot assay used to measure mycobacterial cell wall hydrolase activity. The 1.5% agarose gel contained 0.1mg/ml of FITC conjugated insoluble M. smegmatis cell wall as the matrix. The assay assessed the hydrolase activity of enzyme fractions after ion-exchange chromatography. **1**. Enzyme fraction with hydrolase activity. **2**. Enzyme fraction with only mild hydrolase activity. **3** and **4**. Negative enzyme fractions with no hydrolase activity.

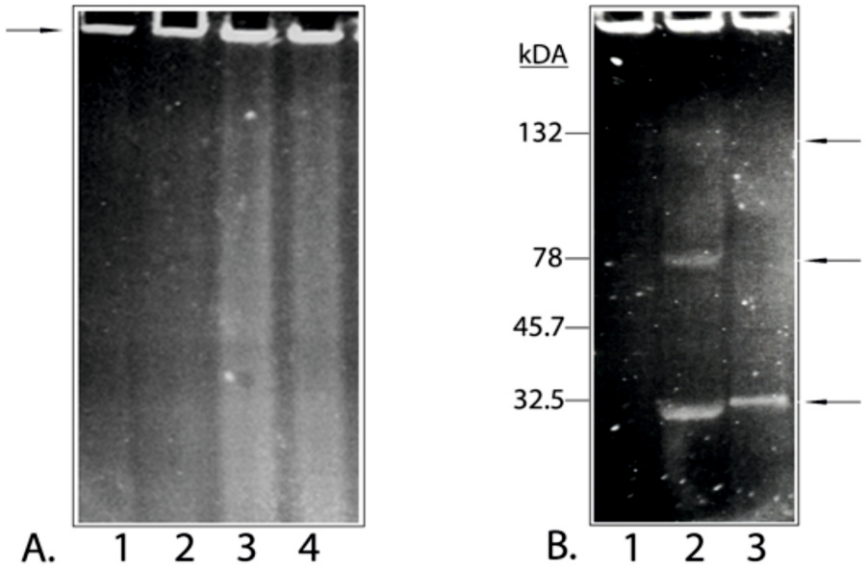


**Figure 2. Identification of mycobacterial cell wall hydrolases and their activities by electrophoretic fluorescent gel.**

**A.** Fluorescent image of an 8-16% native gradient polyacrylamide electrophoresis (PAGE) gel, that contained a reaction mixture of 0.1 mg of lysozyme (active and inactivated) and 2.5 µg of FITC conjugated M. smegmatis cell wall. Lane 1 contained M. smegmatis FITC cell wall and heat-inactivated lysozyme that were incubated for 2 hours. Lanes 2 – 4 contained lysozyme and M. smegmatis FITC cell wall that were incubated for 10 minutes, 2 hours, and 5 hours, respectively. The image was taken using a UV camera without prior incubation. The arrow pointed at fluorescence at the top of each sample lane represented the lysozyme substrate. A fluorescent smear represented the hydrolyzed fluorescent cell wall (with a heterogeneous length of cell wall polymers that were primarily polysaccharides) by enzymatic action.

**B.** Identification of mycobacterial cell wall hydrolases with fluorescent assay and 8-16% gradient PAGE method. Each well was loaded with a hydrolase reaction mixture that contained a partially purified (through ion-exchange and size column chromatography) M. smegmatis hydrolase fraction and 2.5 µg of M. smegmatis FITC cell wall. The mixture was electrophoresed first and then incubated overnight (20 hours) at 37°C. The fluorescence at the top of each lane represented the hydrolase (native M. smegmatis enzyme extracts) substrates (insoluble M. smegmatis FITC cell wall). The bright fluorescent smear in each lane represented the release of soluble cell wall polymers from the insoluble cell wall by enzyme action. The bright fluorescent bands shown by

**Table 1: Sub-cellular distribution of the hydrolytic activity of M. smegmatis cell wall hydrolases.**

| Enzymatic Sample Source | Fluorescence released from FITC labeled M. smegmatis cell wall by M. smegmatis Degradative Enzymes | |
|---|---|---|
| | Fluorescent intensity (495 nm/ 519 nm) | Relative activity (%) |
| Background (no enzyme) | 748 | 0.83% |
| Cytosol | 18401 | 20.4% |
| Cell wall and membrane | 39174 | 43.4% |
| Lysate from total cell pellet | 58365 | 64.6% |
| Cell filtrates (extracellular space) | 32032 | 35.5% |
| Total activity | 89607 | 100% |

Each of the crude enzyme extracts was incubated at 37°C with M. smegmatis FITC cell wall for 75-minutes. The fluorescence was measured using an excitation wavelength of 495 nm and an emission wavelength of 519 nm at 37°C with a microplate reader.